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Chitosan–*N*-poly(ethylene glycol) brush copolymers: Synthesis and adsorption on silica surface

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Abstract

Chitosan–*N*-poly(ethylene glycol) brush copolymers with different degree of substitution (DS) were synthesized via reductive amination of chitosan by methoxy poly(ethylene glycol) (MPEG) aldehyde. Chitosan–*N*-MPEG copolymers were high-molecular-weight products with desirable DS; solubility and solution viscosity of those copolymers depended on the method of the synthesis of MPEG aldehyde and on DS. Synthesis of MPEG aldehyde by the use of TEMPO radical/BAIB was not suitable because of partial oxidation of methoxy groups of MPEG resulting in bifunctional PEG derivatives leading to cross-linking. Adsorption studies of chitosan–*N*-MPEG graft copolymers on silica surface show that these polymers adsorb in highly hydrated layers.

Keywords: Chitosan; Poly(ethylene glycol); Water-soluble; Graft copolymer; Reductive amination; Adsorption, QCM-D, Reflectometry

1. Introduction

Chitosan is a non-toxic, biodegradable, biocompatible, cationic polymer that is derived from naturally occurring polysaccharide, chitin, through a process of deacetylation. Being positively charged, chitosan readily adsorbs on negatively charged surfaces. Due to biodegradability and biocompatibility chitosan is an attractive material in various fields of applications ranging from agriculture and waste-water treatment to pharmaceuticals, food and personal care [1]. However, applications of chitosan are limited by its poor solubility in alkaline solutions. It has been shown [2] that chemical modification of chitosan helps to improve its solubility as well as produces derivatives with new properties.

Hydrophilic charge regulating chitosan–poly(ethylene glycol) graft copolymers are interesting as dispersing agents, solubilization aids, and they are promising as surface conditioners. These polymers are water soluble in a wide pH range and are attractive due to their non-toxicity and biocompatibility [3]. There are nearly a dozen publications concerning chitosan modification through its amino groups by poly(ethylene glycol) (PEG) [2–10]. In most cases methoxy poly(ethylene glycol) (MPEG) was used instead of PEG to avoid cross-linking of copolymers of chitosan. Several publications

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describe synthesis of chitosan–*N*-PEG graft copolymers using MPEG aldehyde as a starting material [3–6]. Harris at al. [3] were the first reporting on the synthesis of PEG–chitosan derivative by the modification of chitosan with reductive alkylation of amino group by PEG aldehyde. Sugimoto et al. [4] prepared PEGylated chitin/chitosan hybrids and studied their solubility. Muslim et al. [5] synthesized chitosan–*N*-PEG hybrids and studied bioactivity of these compounds. Kurita et al. [6] prepared comb-shape chitosan derivatives having tri(ethylene glycol) side chains, which showed significant adsorption capacity toward metal cations. Bentley at al. [7] reported a simple and reliable method for preparation and use in reductive amination of PEG acetaldehyde hydrate generated in situ by hydrolysis of PEG acetaldehyde diethylacetal. Pozzo et al. [8] synthesized PEG dialdehyde diethyl acetals of different molecular sizes and used to generate in situ PEG dialdehydes for the cross-linking of partially reacetylated chitosan. Saito et al. [9] synthesized graft copolymer of MPEG on a chitosan backbone starting with MPEG-*p*-nitrophenyl carbonate and studied the suitability of the PEG-grafted chitosan nanoparticles as a carrier for delivery of anionic drugs such as proteins and oligonucleotides. Ouchi et al. [10] and Ohya et al. [11] grafted carboxy derivatives of MPEG on 6-triphenylmethyl chitosan and studied their aggregation phenomena or usefulness as peptide drug carriers, respectively. Lebouc et al. [12] described the synthesis of chitosan–*N*-MPEG graft copolymers containing amide linkages starting from chitosan or 6-*O*-triphenylmethyl chitosan.

According to the above publications, derivatisation of chitosan with a functionalized PEG resulted in variety of chitosan–*N*-PEG graft copolymers differing in degree of substitution, solubility and molecular weight. However, the degree of substitution of chitosan was rather low in many cases [3,4,6–11], or hydrogels of the graft copolymers were received [4,8,9].

The present work focuses on a detailed study of the synthesis of chitosan brush derivatives by attaching poly(ethylene glycol) grafts at the amino group of the chitosan monosaccharide residue. The effect of preceding oxidation of MPEG to MPEG aldehyde on structure and solubility of the graft copolymers was revealed and discussed. Adsorption properties of the chitosan derivatives on silica surfaces were studied for the first time employing the QCM-D and reflectometry techniques.

2. Experimental

2.1. Materials

2.1.1. Materials for synthesis

Three types of chitosans were used in the study: initial chitosan (chitosan) was purchased from FLUKA (M_r 400,000, degree of deacetylation (DD) 72%). Highly deacetylated chitosan (DA-chitosan, DD 93%) and low-molecular-weight chitosan (LM-chitosan, M_r 6000, DD 84%) were obtained by alkaline and acidic hydrolysis of chitosan, respectively. 2,2,6,6-Tetramethylpiperidin-1-oxyl (TEMPO radical) and [bis(acetoxy)iodo]benzene (BAIB) were obtained from ALDRICH. Methoxy poly(ethylene glycol) (MPEG) was purchased from FLUKA (M_r 2000). Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) were distilled under reduced pressure from CaH_2 powder and stored over molecular sieves (3 Å). All other solvents were of *puriss* grade and used without further purification.

2.1.2. Materials for adsorption studies

The sodium chloride, with >99.5% purity, and hydrochloric acid (pro analysi) (30%), were obtained from Merck and used as received. The water was first pre-treated with a Milli-RO 10 Plus system and further purified with a Milli-Q PLUS 185 system. Ethanol with purity >99.5% was purchased from Kemetyl. The QCM-D sensor chips were purchased from Q-Sense (Göteborg, Sweden). The crystals used were AT-cut quartz crystals (0.3 mm in thickness) with gold-plate electrodes, coated with silica through evaporation. The silica surfaces for QCM-D measurements were cleaned in a Deconex 11 (from Göteborgs Termometerfabriken) solution for 1 h. They were then thoroughly rinsed with Milli-Q water, and ethanol and blow-dried with nitrogen before soaking in Milli-Q water for at least 24 h. Prior to the start of the measurement the crystals were rinsed once more with ethanol and blow-dried with a filtered nitrogen jet.

For reflectometry, thermally oxidized silicon wafers (the ellipsometrically determined thickness of the oxide layer = 100 ± 1 nm) were obtained from Wafer Net, Germany. The wafers were cut to size (1 × 5 cm strips) and conditioned by immersing in a solution mixture of $H_2O/HCl/H_2O_2$ (volume ratios 66:21:13) at 75–80 °C for 10 min. The silica strips were then removed and thoroughly rinsed in Milli-Q water. Next, they were immersed in a solution mixture $H_2O/NH_3/H_2O_2$ (volume ratios 71:17:12) at 75–80 °C for 10 min. Finally, the strips were carefully rinsed in a copious amount of water and stored under purified ethanol. They were removed from the ethanol immediately before use and blow-dried with a filtered nitrogen jet.

2.2. Synthesis procedures

2.2.1. Oxidation of MPEG by the method of "activated" DMSO

Oxalyl chloride (1.4 g, 11 mmol), dry DMSO (1.8 g, 24 mmol) and MPEG (20 g, 10 mmol) were kept over P_2O_5 in a desiccator. The reactants were dissolved in methylene chloride separately. The reaction was carried

out in a four-neck flask equipped with a stirrer, thermometer and two pressure-equalizing additional funnels protected by drying tubes. The solution of oxalyl chloride was cooled down to −30 °C in the flask under stirring. Then the DMSO solution was added dropwise and the mixture was stirred for 10 min. Subsequently the MPEG solution was added dropwise, and the mixture was stirred for additional 15 min. Finally, triethylamine (5.05 g, 50 mmol) was added under stirring at −30 °C. The reaction mixture was filtered, the filtrate was poured into hexane, and the obtained white precipitate was washed several times with diethyl ether and dried in an air. In total 18.4 g of the product was received (yield 92%).

Oxidation of MPEG using acetic anhydride instead of oxalyl chloride was performed using the method described earlier [3–5].

2.2.2. Oxidation of MPEG by the use of TEMPO radical

The three-neck reaction flask, fitted with a stirrer, pressure-equalizing dropping funnel and a thermometer, was charged with MPEG (10 g, 5 mmol), TEMPO radical (7.85 mg, 0.05 mmol) and potassium bromide (59.5 mg, 0.5 mmol) dissolved in the mixture of methylene chloride and water (9:1 w/w). The reaction mixture was vigorously stirred and cooled to −10 °C. A freshly prepared 1 M aqueous solution of sodium hypochlorite (55 ml, 55 mmol, pH 9.5) was dropped in for 15–20 min, keeping the temperature of the reaction mixture between 10 and 15 °C. The mixture was stirred for additional 3 min; the organic phase was separated and washed with 10% aqueous hydrochloric acid containing KI (10 mmol) to remove residual TEMPO radical. The organic phase was reseparated and washed with 10% aqueous sodium thiosulfate solution. The third time the reseparated organic phase was poured into diethyl ether, and the precipitated product was filtered out and dried in an air. In total 9.6 g of the product was received (yield 48%).

Oxidation of MPEG using TEMPO radical in the presence of BAIB was done by the method described elsewhere [13].

2.2.3. Oxidation of MPEG by the use of alcohol oxidase

Alcohol oxidase from the yeast *Pichia pastoris* was used as an enzymatic catalyst [14]. The activity of an aqueous solution of the enzyme was 7.3 U/ml and the activity of the enzyme immobilized on macroporous cellulose was 2.4 U/g. One unit (U) of alcohol oxidase activity was defined as the amount of enzyme that causes the oxidation of 1 μmol of methanol per 1 min at 30 °C and pH 7.3 [15].

Enzyme solution (1 ml) was added to 20 ml of MPEG solution (3–5%) in 0.1 M phosphatic or TRIS buffer (pH 7.3) and the reaction mixture was kept at 35 °C under stirring. Periodically 0.6 ml of the reaction mixture was taken out for immediate spectroscopic determination of the amount of aldehyde groups.

In an alternative procedure 0.7 g of an immobilized enzyme was added to 20 ml of MPEG solution (3–5%) in 0.1 M phosphatic buffer (pH 7.3), and the reaction mixture was kept at 35 °C under stirring. Periodically 2 ml of the reaction mixture was taken out, filtered and 0.6 ml of each solution was used for immediate spectroscopic determination of the amount of aldehyde groups.

2.2.4. Synthesis of N-MPEG chitosan derivatives (an example of the synthesis of chitosan derivative with the degree of substitution 23%)

Chitosan (0.5 g, 2.9 mmol) as a 0.5% solution in 0.4% aqueous acetic acid and MPEGA (2.9 g, 1.45 mmol) as an aqueous 10% solution were placed into a flask fitted with a mechanical stirrer. pH of the reaction mixture was increased by gradually adding Na_2CO_3 until pH 5–6. After 1 h $NaCNBH_3$ (0.183 g, 2.9 mmol) was added and the mixture was stirred for 5 h at 55 °C. The precipitate was obtained by pouring the reaction mixture into a saturated ammonium sulfate solution, collected and dialyzed against water for 96 h using visking dialysis tubing (SERVA). The solution was concentrated using a rotating evaporator until a solid residue was formed. The product was dried in a vacuum oven at 40 °C to give 1.63 g of chitosan–*N*-MPEG graft copolymer (yield 89%).

Anal. Calcd for $[C_{97}H_{193}O_{49}N]_{23}[C_6H_{11}O_4N]_{49}$-$[C_8H_{13}O_5N]_{28}$: C, 52.25; N, 2.22; H, 8.46. Found: C, 49.75; N, 1.90; H 8.73.

The data of elemental analysis suggest that the sample contains about 5% of moisture.

^{1}H NMR spectrum (graft copolymer in D_2O): $\delta = 4.89$ (H-1), $\delta = 3.78$ (H-3, H-4, H-6′), $\delta = 3.58$ (–O–CH_2–), $\delta = 3.23$ (–OCH_3), $\delta = 2.99$ (H-2), $\delta = 1.95$ (–$COCH_3$).

2.3. Analytical procedures

2.3.1. Determination of the degree of conversion of MPEG to MPEG aldehyde

The degree of conversion of MPEG to MPEG aldehyde (DC, %) is expressed as an average number of aldehyde groups per 100 molecules of the oxidized MPEG.

The amount of MPEG aldehyde was evaluated by a spectrophotometric assay using alkaline 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole reagent (PURPALD) [16]. Standard solution of acetaldehyde was prepared by oxidation of L-rhamnose with periodate. 0.6 ml of aqueous solution of MPEGA was added to 0.9 ml of 1% PURPALD solution in 1 M aqueous NaOH, the mixture was incubated on a rotary shaker at room temperature for 30 min, and 1.5 ml of 0.2% alkaline borohydride solution was added under mixing.

The solution was read spectrophotometrically against a reagent blank at 542 nm.

2.3.2. Determination of the degree of deacetylation of chitosan

The degree of deacetylation of chitosan (DD, %) was calculated according to the content of primary amino groups [17] and the content of nitrogen in chitosan determined experimentally by potentiometric titration and elemental analysis respectively.

Knowing amount of primary amino groups (A, %) and nitrogen content (B, %) in a chitosan sample, the following equation is used to calculate DD (%):

$$\mathrm{DD} = \frac{14 \cdot A}{16 \cdot B} \cdot 100, \tag{1}$$

where 14 and 16 are molecular weight of nitrogen and primary amino group respectively.

2.3.3. Determination of the degree of substitution of chitosan

The degree of substitution of MPEG to the monosaccharide residues of chitosan (DS, %) was calculated according to the content of primary amino groups and the content of PEG units in the copolymers determined experimentally.

The content of primary amino groups was determined by a spectrophotometric assay [18]. A copolymer (0.1 g) was accurately weighed and dissolved in 25 ml of 0.1 M phosphate buffer (pH 7.5). 2 ml of the polymer solution was incubated with 0.5 ml of 0.35% aqueous solution of 2,4,6-trinitrobenzoic acid for 15 min at 65 °C. The reaction was stopped by adding 1 ml of 1.5 M formalin solution. A blank experiment was carried out under the same conditions. The solution was read spectrophotometrically against a reagent blank at 420 nm. The content of amino groups (a, %) in a copolymer was calculated as follows:

$$a = \frac{C \cdot D \cdot 16}{m} \cdot 100, \tag{2}$$

where C—the concentration (mol/25 ml) of amino groups determined from calibration curve; 16—the molecular weight of amino group; m—the sample weight, g; D—the dilution ratio.

The content of PEG units in a copolymer (b, %) was determined by a colorimetric method based on the partitioning of a chromophore present in an ammonium ferrothiocyanate reagent from the aqueous to a chloroform phase in the presence of PEG [19].

Knowing a from Eq. (1) and b from the colorimetric data, the following equation is used to calculate DS (%):

$$\mathrm{DS} = \frac{\dfrac{b}{2000}}{\dfrac{b}{2000} + \dfrac{a}{16} + \dfrac{100 - \dfrac{2143 \cdot b}{2000} - \dfrac{161 \cdot a}{16}}{203}} \cdot 100, \tag{3}$$

where 2143—the molecular weight of chitosan monosaccharide residue containing MPEG-2000; 161—the molecular weight of glukosamine monosaccharide residue; 203—the molecular weight of N-acetylated monosaccharide residue; 2000—the molecular weight of MPEG-2000.

2.3.4. IR and NMR spectroscopy, viscometry and determination of molecular weight

The infrared absorption spectra were recorded with a PERKIN ELMER Spectrum BX spectrometer under dry air at 20 °C by the KBr pellet method. The ^{1}H NMR spectra of copolymers were recorded on a UNITY INOVA VARIAN spectrometer (300 MHz, Varian). The samples were prepared in D_2O or D_2O containing one drop of DCl.

The molecular weight of the DA-chitosan–N-MPEG graft copolymer (DS 89%) was determined by GPC using Wyatt DAWN EOS (light scattering) and RI detectors. The columns used were Ultrahydrogel linear + TSK GMP2000, eluent aqueous (0.3 M $NaNO_3$ + 2 ml/l 1 M NaOH), pH ~ 10, eluent flow rate 0.8 ml/min, concentration approximately 1 mg/ml.

The intrinsic viscosity of the copolymer solutions in aqueous 0.5 M CH_3COOH/0.5 M CH_3COONa at 25 °C was measured using a dilution-type Ubbelohde viscometer.

2.4. Adsorption of graft copolymers on silica surfaces

The adsorption properties of chitosan and chitosan derivatives were studied employing Quartz Crystal Microbalance with Dissipation (QCM-D) and reflectometry techniques.

2.4.1. Quartz crystal microbalance with dissipation

The QCM-D (Q-Sense AB, Göteborg, Sweden), allows simultaneous detection of the changes in the resonance frequency (Δf) and dissipation factor (ΔD). The principles behind this technique are thoroughly described by Rohdal et al. [20]. In short, the utilization of the crystal as a sensitive balance is based on the fact that any change in crystal mass, Δm, causes a shift in resonance frequency, Δf. If the attached mass is evenly distributed, rigidly attached and small, compared to the mass of the crystal, the value of Δf can be related to the mass per surface unit (Δm) by the Sauerbrey equation [20]:

$$\Delta m = \frac{C \cdot \Delta f}{n}, \tag{4}$$

where n—the overtone number ($n = 1, 3, 5, 7$); Δf—the resonant frequency ($f_0 \approx 5$ MHz); C—the constant that describes the sensitivity of the instrument to changes in mass ($C = 0.177$ mg m^{-2} Hz^{-1}).

Energy dissipation occurs due to losses within the adsorbed layer and due to coupling with the solution. Hence, when the driving voltage is turned off, a damping of the oscillations occurs. The decay rate of the amplitude reflects the energy dissipation and it is affected by the viscoelastic properties of the material. The dissipation factor, D, is defined by equation [20]:

$$D = \frac{E_{\text{diss}}}{2 \cdot \pi \cdot E_{\text{stor}}}, \quad (5)$$

where E_{diss}—the total dissipated energy during one oscillation cycle, E_{stor}—the total energy stored in the oscillation.

2.4.2. Reflectometry

The adsorbed amount of DA-chitosan–*N*-MPEG was determined by fixed angle reflectometry in combination with a stagnation point flow cell. The principles behind this technique along with the experimental set-up used in this study have been thoroughly described by Dijt et al. [21]. In our evaluation of DA-chitosan–*N*-MPEG adsorbed amount we assumed $dn/dc = 0.136$ ml/g, the value for PEG in water [22]. We justify our choice of dn/dc by considering that the PEG grafts account for approximately 92% of a molecular weight of DA-chitosan–*N*-MPEG.

2.4.3. Sample preparation

Polyelectrolyte solutions for adsorption studies were prepared by dissolving polyelectrolytes to 50 ppm (w/w) in pH adjusted (pH 5–6) 0.1 mM NaCl solutions. The solutions were allowed to stabilise for approximately 24 h. Finally, if necessary, before carrying out the adsorption experiment, the pH was readjusted to 5–6. The experiments were in all cases carried out at 25 °C.

3. Results and discussion

The pathway of N-PEGylation of chitosan by the use of reductive amination is presented in Scheme 1. Reductive amination is the reaction between amine and aldehyde groups in the presence of a reducing agent. Reductive amination using $NaCNBH_3$ is known as the Borch reaction [23]. The Borch reaction requires a protic solvent or addition of an equivalent amount of an acid. Keeping in mind that chitosan is soluble in acidic media only these conditions are very suitable for modification of chitosan.

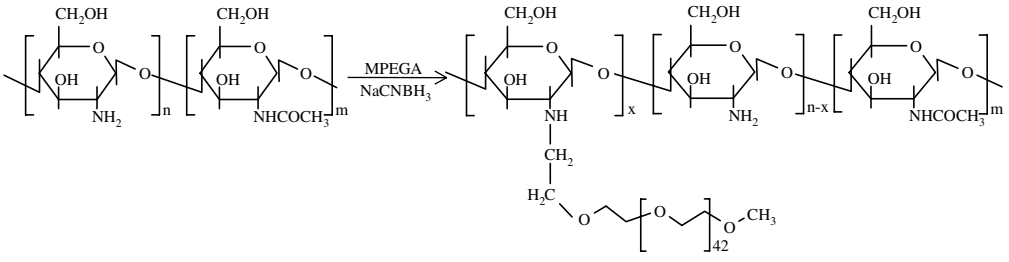
3.1. Oxidation of MPEG to MPEG aldehyde

Several methods have been described in the last few decades to convert free terminal hydroxyl groups of PEG into aldehyde. These electrophilic derivatives of PEG have mostly been obtained by direct oxidation of the primary alcohol carbon of the oligomer [3,11,12,24]. The oxidation of PEG with DMSO/acetic anhydride (Moffatt oxidation) described by Harris et al. [11] was among the preferred methods. Unfortunately, the degree of conversion, defined as the ratio of the amount of the product carbonyl groups to that of the original hydroxyl groups, usually did not exceed 50–55% [11].

In this study we examined the synthesis of MPEGA using five different methods; four of them were chemical and one enzymatic. Two of the chemical methods were based on the use of TEMPO radical in the presence of NaClO [25] or BAIB. The other two were oxidation by "activated" DMSO [26], using different "activators", i.e. acetic anhydride as described earlier [3,11], or oxalyl chloride in a similar way as used for oxidation of primary alcohols in organic chemistry [26]. The enzymatic method is based on selective oxidation catalysed by the enzyme *alcohol oxidase*, and it is applied for the first time for oxidation of poly(ethylene glycol).

The results of MPEG oxidation by the different methods are summarized in Table 1. Using oxalyl chloride as an "activator", DC of MPEG to MPEGA under optimal conditions reached 70%. It was determined that low temperature (−30 to −35 °C) of the reaction and the dryness of the reaction components had a noticeable effect on the DC [26]. Even small amount of water in the reaction mixture (e.g., moisture in MPEG) actuated oxidation to carboxy groups instead of aldehyde.

Moderate DC of MPEG to MPEGA was achieved using the catalytic procedure employing TEMPO radical



Scheme 1. Modification of chitosan by methoxy poly(ethylene glycol).

Table 1
Oxidation of MPEG by different methods

Oxidizing method		pH	*T*, °C	Yield, %	DC, %
"Activated" DMSO	Acetic anhydride		20	90	30
	Oxalyl chloride		−30	92	70
			−30	90	55[a]
			−25	88	45
TEMPO radical	NaClO	12.7	20	48	31
		9.8	20	46	49
		9.5	20	50	54
		9.5	20	49	32[b]
	BAIB		20	92	110
			35	87	128
Alcohol oxidase	In solution	7.3	35	–	<1
	Immobilized	7.3	35	–	<1

[a] Molar ratio of oxalyl chloride to DMSO 1:2.7.
[b] Molar ratio of MPEG to NaClO 1:22.

in the presence of NaClO (Table 1). The DC depended on the ratio of NaClO to TEMPO radical, but the maximal DC did not exceed 54%. This is in agreement with the recent investigations of Vigo et al. [27] who concluded that none of the methods gave satisfactory results for the carboxymethyl end capping of PEG's. Moreover, the yield of the obtained MPEGA, which depends on loss of the materials under isolation and purification, was not higher than 50%.

Oxidation of MPEG by TEMPO radical in the presence of BAIB resulted in very high DC (Table 1). Surprisingly, in some cases the DC was even higher that 100%. It was rationalized by suggesting that a part of the methoxy groups present in MPEG was oxidized to aldehyde as well. If this is the case, some PEG molecules containing two aldehyde groups should be formed. That this indeed was the case was proved later when using MPEGA from different synthesis for grafting on chitosan. Partly cross-linked chitosan–2-*N*-MPEG graft copolymers were received in one case only, and that was when MPEGA synthesized in the presence of BAIB was used.

An attempt to use enzymatic method for oxidation of MPEG was not successful. The oxidation rate was high at the beginning but it was drastically decreasing during the course of the reaction. It was determined that denaturation of the enzyme took place which was caused by the end products of the reaction MPEGA and hydrogen peroxide [28]. Thus, the conditions for oxidation of MPEG by *alcohol oxidase* are not ascertained at the present.

Summarizing the results of oxidation, we conclude that none of the methods used gives a DC of MPEG to MPEGA close to 100%. Keeping in mind that solubility of the succeeding graft copolymers is a very important parameter, the method of "activated DMSO" using oxalyl chloride as activator was chosen as the main route for the synthesis of MPEGA.

3.2. Synthesis of chitosan–N-MPEG graft copolymers

Procedures of the synthesis of chitosan–*N*-MPEG graft copolymers starting from chitosan, DA-chitosan and LM-chitosan were identical. In all the cases the reducing agent was added to the reaction mixture after 1 h from the beginning to avoid precipitation of chitosan due to high alkalinity of $NaCNBH_3$. The results of N-PEGylation of chitosans are summarized in Table 2. According to chemical analysis data, the obtained chitosan–*N*-MPEG graft copolymers contained large amount of PEG units and predicted amount of free amino groups. It was determined that the DS of chitosan almost linearly depended on the molar ratio of MPEGA to chitosan monosaccharide residue. The reaction between chitosan amino groups and MPEGA in the presence of the reducing agent was irreversible and proceeded until consumption of the active functional groups. Using equimolar amount of MPEGA and chitosan monosaccharide residue (entry no. 5 in Table 2), DS nearly equal to DA of chitosan was achieved. The use of DA-chitosan (DA 93%) enabled to increase DS up to 89% (entry no. 7 in Table 2) resulting in the brush copolymer with very high density of MPEG grafts. At a lower ratio of MPEGA to chitosan monosaccharide residue, chitosan derivatives with different degree of *N*-substitution were obtained, providing a versatile synthetic route for controlling the graft density of these brush copolymers.

The reaction of reductive N-PEGylation of chitosan is very fast. It was determined studying N-PEGylation

Table 2
The results of the analysis of *N*-PEGylated chitosans

No.	Compound	Amount of		DS, %	[η]	Solubility in water
		PEG, %	NH_2, %			
1	MPEGA	100	–	–	0.08	+
2	Chitosan	–	6.6	–	8.42	–
3	Chitosan–*N*-MPEG	73	1.25	23	2.95	+[a]
4	Chitosan–*N*-MPEG	87	0.20	56	0.98	+
5	Chitosan–*N*-MPEG	90	0.01	71	0.29	+
6	DA-chitosan	–	8.41	–	7.55	–
7	DA-chitosan–*N*-MPEG	94	0.04	89	0.29	+
8	LM-chitosan	–	8.0	–	0.33	–
9	LM-chitosan–*N*-MPEG	81	1.15	35	0.20	+
10	LM-chitosan–*N*-MPEG	89	0.25	62	0.25	+
11	LM-chitosan–*N*-MPEG	91	0.04	80	0.28	+

[a] Under heating.

of DA-chitosan that DS ca. 70% was obtained within 5 min from the beginning of the reaction. Further increase in DS is much slower, and the reaction usually lasts several hours to reach predicted DS.

Grafting of MPEG on chitosan was confirmed by FT-IR spectra. FT-IR spectra of chitosan–*N*-MPEG brush copolymers showed the absorption bands characteristic both for chitosan and MPEG with much higher absorbance of the groups of the latter. This is consistent with the fact that PEG chains are long (M_r 2000, about 44 ethylene oxide units). Distinctive absorption bands of PEG at 1110 cm^{-1} (C–O stretching) and 2886 cm^{-1} (C–H stretching) were present in the spectra of the brush copolymers irrespective of DS. On the contrary, two bands of chitosan at 1650 cm^{-1} and 1560 cm^{-1} assigned to the amide-I and amide-II groups respectively almost disappeared in the spectra of the copolymers with high DS. Due to relatively high-molecular weight of MPEG (2000), ^1H NMR spectra of chitosan–MPEG graft copolymers provide little information on the structure of the copolymers. Strong broad peak of oxymethyl groups of PEG at 3.45–3.7 ppm prevails in the spectra and partially cover over the signals of the pyranose ring of chitosan. Nevertheless in the spectrum of a chitosan derivative with relatively low DS (Fig. 1) the signals of chitosan are seen ($\delta = 1.95$ ($-COCH_3$), $\delta = 2.99$ (H-2), $\delta = 3.78$ (H-3, H-4, H-6′)) and prove that the studied samples are copolymers.

Separation and purification of chitosan–*N*-MPEG brush copolymers is a serious problem. The use of a membrane dialysis was found ineffective, and the dialyzed product contained a part of unreacted MPEG. This is consistent with the earlier findings of Sugimoto et al. [4] claiming that PEG could not be separated from the mixture of PEG, chitosan and chitosan–*N*-PEG copolymer by a membrane dialysis. Precipitation of the brush copolymers with acetone as reported by Muslim et al. [5] was found reasonable if DS of chitosan was low. It was found in our study that the salting out with aqueous saturated ammonium sulfate solution helped to separate chitosan–*N*-MPEG brush copolymers with high DS. Chitosan–*N*-MPEG copolymers formed concentrated gel-like upper phase under salting out and were removed while unbound MPEG remained in the solution. The purification procedure of the graft copolymers was ended by membrane dialysis against water to remove residual ammonium sulfate.

Reductive N-PEGylation of LM-chitosan is very similar to that described above. However, separation and purification of the obtained graft copolymers is more complicated in this case. These copolymers were dialyzed against water first to remove $NaCNBH_3$, then dried and washed with acetone to remove residual unbound MPEG. Unfortunately, a part of these copolymers dissolved under washing with acetone resulting in lower yield of the products.

Solubility results (Table 2) suggest that degree of substitution and molecular weight of the copolymers have some effect on solubility properties of the copolymers. Chitosan–*N*-MPEG brush copolymers with high DS are easily soluble in water. Graft copolymers with relatively low DS (<40%) could be dissolved under heating at 40–50 °C only. LM-chitosan–*N*-MPEG graft copolymers dissolve much faster than those obtained from high-molecular weight chitosan. Chitosan–*N*-MPEG graft copolymers become soluble in water and weakly alkaline aqueous solutions when the DS reaches about 20%.

The intrinsic viscosity of aqueous solutions of the graft copolymers strongly depends on the DS (Table 2). Solutions of chitosan derivatives with low DS are significantly more viscous than the solutions of copolymers with high DS (see Table 2). It is well known that the viscosity of linear polymers depends on molecular weight. However, brushes of graft copolymers make macromolecules stiffer and distort this dependence. Thus, the low

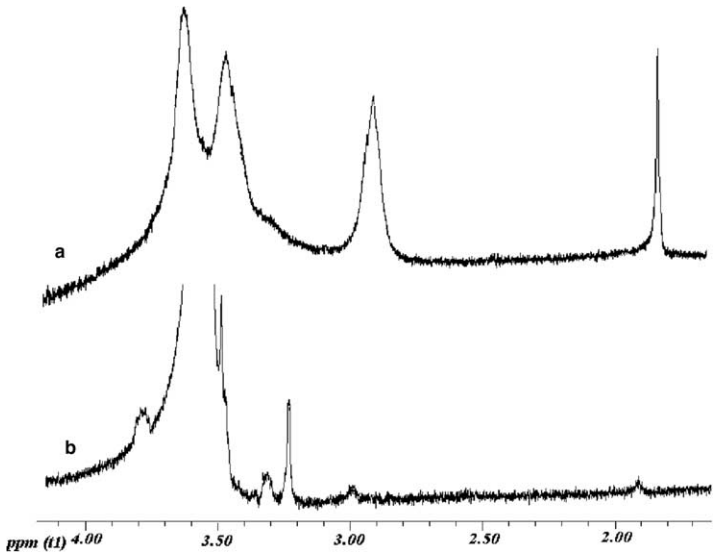


Fig. 1. Fragment of ^{1}H NMR spectra of chitosan in D_2O (containing one drop of DCl) (a) and chitosan–*N*-MPEG brush copolymer with DS 23% in D_2O (b).

solution viscosity of a highly modified chitosan does not mean that the molecular weight of this copolymer is low. Analysis of the DA-chitosan–*N*-MPEG copolymer (DS 89%) by the GPC technique revealed that this chitosan derivative with relatively low solution viscosity (Table 2) possessed high-molecular weight (M_w 830,000, M_n 450,000). The lower compare with chitosan viscosity of aqueous solutions of chitosan–*N*-MPEG copolymers could be explained, probably, by the properties of MPEG. It is known that in an aqueous medium a long chain of PEG molecule is heavily hydrated and the presence of grafted PEG chains counteracts association. Thus, PEG can be thought of as a ''molecular windshield wiper'' [17]. When this molecular windshield wiper is attached to a molecule, it prevents the approach of other molecules thus preventing association or hydrogen bonding.

3.3. Adsorption of chitosan–MPEG graft copolymers on silica surfaces

We used QCM to compare the adsorption properties of the initial chitosan and two chitosan derivatives chitosan–*N*-MPEG PEGylated to 56%, and DA-chitosan–*N*-MPEG with degree of PEGylation 89%. After obtaining a stable baseline in the background electrolyte solution (0.1 mM NaCl), the samples were injected into the measuring chamber (0.5 ml) and the adsorption was followed for 1.5 h before the chamber was rinsed with 5 ml of the background solution. After this the changes in frequency and dissipation were monitored for 30 min. In Table 3, the sensed mass and dissipation data demonstrate the reproducibility of the measurements.

Table 3
Adsorption of chitosan and its derivatives on silica surfaces in 1.5 h

Polymer	Δm (mg/m^2)	$\Delta D \times 10^{-6}$
Chitosan	1.1 ± 0.2	1.4 ± 0.1
Chitosan–*N*-MPEG	8.9 ± 2.7	7.8 ± 0.9
DA-chitosan–*N*-MPEG	9.5 ± 1.7	8.4 ± 0.8

It is important to note that the resonant frequency is related to the total oscillating mass, thus detection of changes in resonance frequency allows determination of a sensed mass rather than an adsorbed mass. The sensed mass includes the mass of both the adsorbate and the trapped solvent. This constitutes an important difference from the reflectometry technique, which was also used in this study, where the adsorbed amount of the adsorbate alone is detected.

From Fig. 2 it is seen that the adsorbed amount of chitosan on the silica surface is relatively low. The sensed mass is just below 1 mg/m^2. The dissipation is also low (see Fig. 2), showing that chitosan adopts flat conformations on the silica surface. A consequence of this is that the polymer chain occupies a large area on the surface. Adsorption of chitosan–*N*-MPEG results in about five times larger sensed mass and significantly larger dissipation as compared to its unmodified counterpart. This suggests that the modified chitosan adsorption layer is much less compact than the layer of chitosan. The adsorbed layer of DA-chitosan–*N*-MPEG appears to be rather similar to that formed by chitosan–*N*-MPEG. It is clear that the graft density of the poly-

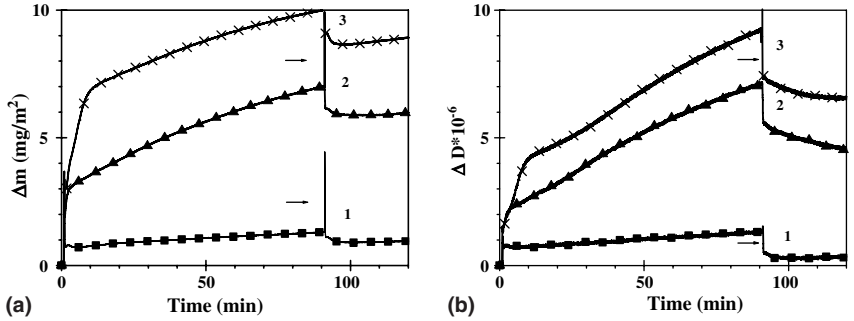


Fig. 2. The adsorption of chitosan (1), chitosan–*N*-MPEG (2) and DA-chitosan–*N*-MPEG (3) in terms of sensed mass Δm (a) and dissipation change ΔD (b) measured with QCM-D. The arrows indicate the start of rinsing of polyelectrolyte adsorption layers with 0.1 mM NaCl solution.

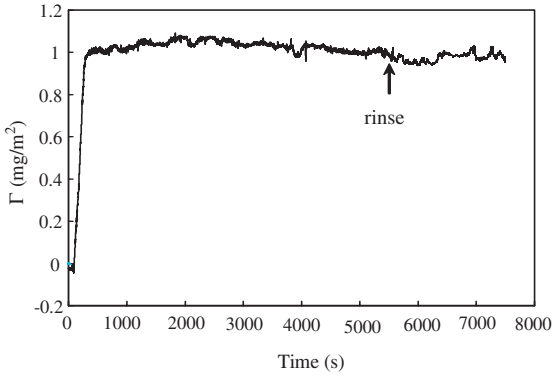


Fig. 3. Adsorbed amount of DA-chitosan–*N*-MPEG on silica as a function of time measured by reflectometry.

mer has a major impact on its adsorption properties. The adsorption layers of PEG modified chitosans are heavily hydrated. From the direct comparison of adsorption data obtained by the QCM-D (Fig. 2) and reflectometry (Fig. 3) (compare adsorption plateau values after rinsing) we can infer that the water content in the adsorption layer of DA-chitosan–*N*-MPEG amounts to about 7.9 mg/m^2, which equals to close to 89% of the sensed mass.

We note that DA-chitosan–*N*-MPEG has both an electrostatic affinity to the negatively charged silica surface due to the presence of charged amino groups, and a non-electrostatic affinity due to the PEG grafts that adsorb on silica surface via hydrogen bonding [29]. However, due to steric hindrance it is not favourable for the MPEG-side chains to accumulate at the surface. Hence, by increasing the degree of substitution it becomes more difficult for the polymer chain to adsorb in flat conformations. This is demonstrated by the high dissipation values. This conclusion is also strongly supported by reflectometry data. Whereas adsorption of unmodified chitosan on the silica surface results in an adsorbed amount of 0.2 mg/m^2, adsorption of DA-chitosan–*N*-MPEG from 0.1 mM NaCl solution leads to an adsorbed amount of 1 mg/m^2 (Fig. 2). This data, in terms of adsorbed saccharide units yields 6.5×10^{17} and 2.9×10^{17} saccharide units per square meter for chitosan and DA-chitosan–*N*-MPEG, respectively. Thus, in terms of saccharide units, there is only half as much DA-chitosan–*N*-MPEG adsorbed on the surface as compared to its non-modified counterpart.

4. Conclusions

Chitosan–*N*-poly(ethylene glycol) brush copolymers with different degree of substitution (DS) and molecular weight were synthesized via reductive amination of chitosan by methoxy poly(ethylene glycol) (MPEG) aldehyde. Chitosan–*N*-MPEG copolymers were high-molecular-weight products with desirable DS; solubility and solution viscosity of those copolymers depended on the method of the synthesis of MPEG aldehyde and on DS. Synthesis of MPEG aldehyde by the use of TEMPO radical/BAIB was not suitable because of partial oxidation of methoxy groups of MPEG resulting in bifunctional PEG derivatives leading to cross-linking. Adsorption studies of chitosan–*N*-MPEG graft copolymers on silica surface show that these polymers adsorb in highly hydrated layers.

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